POSTWEANING MULTISYSTEMIC WASTING SYNDROME VIRUS FROM PIGS

Technical Field

The present invention relates generally to viruses. More particularly, the present invention pertains to the isolation and characterization of a new PCV isolates, including PMWS 412, from pigs infected with postweaning multisystemic wasting syndrome.

Disclosure of the Invention

The postweaning multisystemic wasting syndrome (PMWS) of pigs is a newly emerged disease, which appears to destroy the host immune systems. A new virus, PMWS 412, was isolated from the homogenized tissue of a PMWS affected piglet. This virus shares many common features with the nonpathogenic porcine circovirus (PCV) isolated from persistently infected PK15 cells. We have cloned and sequenced this novel PCV variant, isolated from the PMWS pig, as well as several additional isolates of the new PCV. These viral clones and sequences are useful for future diagnosis of PMWS and vaccine development.

This is the first time that a virus isolated from PMWS-affected pigs has been cloned and completely sequenced. The new viral genome shares 76% identity with PCV isolated from infected PK15 cells at the nucleotide level. Nucleotide insertions and deletions (indels) were found in three regions. The new virus contains at least six potential open reading frames (ORFs) encoding proteins larger than 50 amino acid residues while PCV

derived from PK15 has seven potential ORFs. Four of the ORFs of these two viruses are somewhat homologous to each other, and the identities at the protein level are 83.5%, 58.9%, 40.9% and 29.1%. The highest homology is seen in the putative DNA replicase gene.

PMWS is a newly emerged disease which causes a high mortality rate in weaned pigs. This disease has a long incubation period, typically 7-8 weeks, and affects many organs of infected pigs. Previous diagnosis of the disease has been based solely on histopathological examination. The porcine circovirus causes worldwide infection in swine and is highly contagious. However, no pathologenic effect was shown by PCV isolated from persistently infected PK15 cells when the virus was used to infect experimental animals, although this virus can infect macrophage cells *in vitro*. We have been able to reproduce the PMWS using tissues collected from PMWS-affected pigs. The novel circovirus, PMWS 412, was isolated from tissue of experimentally infected PMWS pigs. Other novel isolates, 97-41 and B9, have now been isolated from naturally infected piglets. The new PCV is different from that isolated from persistently infected PK15 cell lines.

The main cellular target for the field PCV is the mononuclear cells in the peripheral blood, most possibly the macrophage cells, although the virus is also found in various organs. The affected macrophages lose their normal function and therefore damage the host immune systems leading to death. PMWS affected piglets often die from respiratory failure and interstitial pneumonia with histiocytic cell infiltration. Some members in the circovirus family have been shown to cause anemia and immunodeficiency-related diseases. Our results indicate that PCV can be first detected in the

peripheral blood mononuclear cells of the experimentally infected piglets.

The cloning and sequencing of the novel circoviruses provides information about the causative agent of PMWS. The sequencing information, the clones and its gene products are useful for diagnosis and in vaccine development. In particular, PCR and antibody-based diagnostic methods are useful in the diagnosis of the disease and were used herein to specifically identify and differentiate this novel PCV from PCV derived from persistently infected PK15 cells. The sequencing information is also useful in the design of specific primers, to express viral-specific gene products, to study the viral structure, to generate specific antibodies and to identify virulent genes in porcine circovirus-related diseases.

For example PCV proteins and fragments thereof, antibodies thereto, and genes coding therefor, can be used as diagnostic reagents to detect the presence of infection in a mammalian subject. Similarly, the genes encoding the proteins can be cloned and used to design probes to detect and isolate homologous genes in other viral isolates. For example, fragments comprising at least about 15-20 nucleotides, more preferably at least about 20-50 nucleotides, and most preferably about 60-100 or more nucleotides, will find use in these embodiments.

This is the first time a circovirus was cloned from viral particles instead of replicated form of DNA. By comparing the sequencing and structural similarity among the viruses in the circovirus family, a unique primer was designed taking advantage of the complementary sequences of a conserved stem loop structure. One primer PCR was then performed and the products cloned. Two full-length viral genomes in different orientations inserted into the plasmid vector were completely

sequenced in both directions. Additional PCR products were made and sequenced to ensure the fidelity of the primer/stem loop region.

Brief Description of the Figures

Figure 1 is the nucleotide sequence for PMWS Clone 412.

Figure 2 is a diagram of PMWS 412, showing the location of the open reading frames.

Figure 3 is a diagram of PCV, showing the location of the open reading frames.

Figures 4A-4C are a comparison of the PMWS 412 genome and the PCV genome, showing regions of homology.

Figures 5A-5D are comparisons of amino acid sequences from open reading frames of PMWS 412 versus PCV.

Figures 6A-6B are comparisons of the nucleotide sequences of various PCV isolates.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA technology, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Vols. I, II and III, Second Edition (1989); *DNA Cloning*, Vols. I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds. 1984); *Animal Cell Culture* (R.K. Freshney ed. 1986); *Immobilized Cells and Enzymes* (IRL press, 1986); Perbal, B., *A Practical Guide to Molecular Cloning* (1984); the series, *Methods In Enzymology* (S. Colowick and N.

Kaplan eds., Academic Press, Inc.); and *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., 1986, Blackwell Scientific Publications).

All publications, patents and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety.

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise.

The terms "PCV protein" or a nucleotide sequence encoding the same, intends a protein or a nucleotide sequence, respectively, which is derived from a novel PCV isolate, as described herein. The nucleotide sequences and corresponding amino acid sequences for several PCV isolates are shown in Figures 4A-4C, Figures 5A-5D and Figures 6A-6B. However, a PCV protein or a gene encoding the same, as defined herein is not limited to the depicted sequence.

Furthermore, the derived protein or nucleotide sequences need not be physically derived from the genes described above, but may be generated in any manner, including for example, chemical synthesis, isolation (e.g., from PCV) or by recombinant production, based on the information provided herein. Additionally, the term intends proteins having amino acid sequences substantially homologous (as defined below) to contiguous amino acid sequences encoded by the genes, which display immunological activity.

Thus, the terms intend full-length, as well as immunogenic, truncated and partial sequences, and active analogs and precursor forms of the proteins. Also included in the term are nucleotide fragments of the particular gene that include at least about 8 contiguous base pairs, more preferably at least about 10-20 contiguous base pairs, and most preferably at least about 25 to 50 or more contiguous base pairs of the gene. Such fragments are useful as probes and in diagnostic methods, discussed more fully below.

The terms also include proteins in neutral form or in the form of basic or acid addition salts depending on the mode of preparation. Such acid addition salts may involve free amino groups and basic salts may be formed with free carboxyls. Pharmaceutically acceptable basic and acid addition salts are discussed further below. In addition, the proteins may be modified by combination with other biological materials such as lipids and saccharides, or by side chain modification, such as acetylation of amino groups, phosphorylation of hydroxyl side chains, oxidation of sulfhydryl groups, glycosylation of amino acid residues, as well as other modifications of the encoded primary sequence.

The term therefore intends deletions, additions and substitutions to the sequence, so long as the polypeptide functions to produce an immunological response as defined herein. In this regard, particularly preferred substitutions will generally be conservative in nature, i.e., those substitutions that take place within a family of amino acids. For example, amino acids are generally divided into four families: (1) acidic -- aspartate and glutamate; (2) basic -- lysine, arginine, histidine; (3) non-polar -- alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar -- glycine,

asparagine, glutamine, cystine, serine threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as aromatic amino acids. For example, it is reasonably predictable that an isolated replacement of leucine with isoleucine or valine, or vice versa; an aspartate with a glutamate or vice versa; a threonine with a serine or vice versa; or a similar conservative replacement of an amino acid with a structurally related amino acid, will not have a major effect on the biological activity. Proteins having substantially the same amino acid sequence as the reference molecule, but possessing minor amino acid substitutions that do not substantially affect the immunogenicity of the protein, are therefore within the definition of the reference polypeptide.

An "isolated" nucleic acid molecule is a nucleic acid molecule separate and discrete from the whole organism with which the molecule is found in nature; or a nucleic acid molecule devoid, in whole or part, of sequences normally associated with it in nature; or a sequence, as it exists in nature, but having heterologous sequences (as defined below) in association therewith.

By "subunit vaccine composition" is meant a composition containing at least one immunogenic polypeptide, but not all antigens, derived from or homologous to an antigen from a pathogen of interest. Such a composition is substantially free of intact pathogen cells or particles, or the lysate of such cells or particles. Thus, a "subunit antigen composition" is prepared from at least partially purified (preferably substantially purified) immunogenic polypeptides from the pathogen, or recombinant analogs thereof. A subunit antigen composition can comprise the subunit antigen or

antigens of interest substantially free of other antigens or polypeptides from the pathogen.

The term "epitope" refers to the site on an antigen or hapten to which specific B cells and/or T cells respond. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site." Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen.

An "immunological response" to a composition or vaccine is the development in the host of a cellular and/or antibody-mediated immune response to the composition or vaccine of interest. Usually, an "immunological response" includes but is not limited to one or more of the following effects: the production of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells and/or $\gamma\delta$ T cells, directed specifically to an antigen or antigens included in the composition or vaccine of interest. Preferably, the host will display either a therapeutic or protective immunological response such that resistance of the mammary gland to new infection will be enhanced and/or the clinical severity of the disease reduced. Such protection will be demonstrated by either a reduction or lack of symptoms normally displayed by an infected host, a quicker recovery time and/or a lowered somatic cell count in milk from the infected quarter.

The terms "immunogenic" protein or polypeptide refer to an amino acid sequence which elicits an immunological response as described above. An "immunogenic" protein or polypeptide, as used herein, includes the full-length sequence of the protein, analogs thereof, or immunogenic fragments thereof. By "immunogenic fragment" is meant a fragment of a protein

which includes one or more epitopes and thus elicits the immunological response described above. Such fragments can be identified using any number of epitope mapping techniques, well known in the art. See, e.g., *Epitope Mapping Protocols* in Methods in Molecular Biology, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, New Jersey. For example, linear epitopes may be determined by e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:3998-4002; Geysen et al. (1986) *Molec. Immunol.* 23:709-715, all incorporated herein by reference in their entireties. Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols, supra.*

Immunogenic fragments, for purposes of the present invention, will usually include at least about 3 amino acids, preferably at least about 5 amino acids, more preferably at least about 10-15 amino acids, and most preferably 25 or more amino acids, of the molecule. There is no critical upper limit to the length of the fragment, which could comprise nearly the full-length of the protein sequence, or even a fusion protein comprising two or more epitopes of the protein.

"Native" proteins or polypeptides refer to proteins or polypeptides isolated from the source in which the proteins naturally occur. "Recombinant" polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. "Synthetic" polypeptides are those prepared by chemical synthesis.

A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A DNA "coding sequence" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory elements. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxy) terminus. A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3′ to the coding sequence.

DNA "control elements" refers collectively to promoters, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell. Not all of these control sequences need always be present in a recombinant vector so long as the desired gene is capable of being transcribed and translated.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control elements operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control elements need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter and the coding sequence and the promoter can still be considered "operably linked" to the coding sequence.

A control element, such as a promoter, "directs the transcription" of a coding sequence in a cell when RNA polymerase will bind the promoter and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous nucleic acid molecule.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In procaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the exogenous DNA.

"Homology" refers to the percent identity between two polynucleotide or two polypeptide moieties. The correspondence between the sequence from one moiety to another can be determined by techniques known in the art. For example, homology can be determined by a direct comparison of the sequence information between two polypeptide molecules by aligning the sequence information and using readily available computer programs such as ALIGN, Dayhoff, M.O. (1978) in *Atlas of Protein Sequence and Structure* 5:Suppl. 3, National biomedical Research Foundation, Washington, DC.

Alternatively, homology can be determined by hybridization of polynucleotides under conditions which form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when the sequences exhibit at least about 80%-85%, preferably at least about 90%, and most preferably at least about 95%-98% sequence identity over a defined length of the molecules, as determined using the methods above. As used herein, substantially homologous also refers to sequences showing complete identity to the specified DNA or polypeptide sequence. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning*, *supra*; *Nucleic Acid Hybridization*, *supra*.

The term "functionally equivalent" intends that the amino acid sequence of a protein is one that will elicit a substantially equivalent or enhanced immunological response, as defined above, as compared to

the response elicited by a reference, or an immunogenic portion thereof.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a viral gene, the gene will usually be flanked by DNA that does not flank the viral gene in the genome of the source virus. Another example of the heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

The term "treatment" as used herein refers to either (i) the prevention of infection or reinfection (prophylaxis), or (ii) the reduction or elimination of symptoms of the disease of interest (therapy).

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from a subject, including but not limited to, for example, blood, plasma, serum, fecal matter, urine, bone marrow, bile, spinal fluid, lymph fluid, samples of the skin, external secretions of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, organs, biopsies and also samples of *in vitro* cell culture constituents including but not limited to conditioned media resulting from the growth of cells and tissues in culture medium, e.g., recombinant cells, and cell components.

As used herein, the terms "label" and "detectable label" refer to a molecule capable of detection, including, but not limited to, radioactive isotopes, fluorescers, chemiluminescers, enzymes, enzyme

substrates, enzyme cofactors, enzyme inhibitors, chromophores, dyes, metal ions, metal sols, ligands (e.g., biotin or haptens) and the like. The term "fluorescer" refers to a substance or a portion thereof which is capable of exhibiting fluorescence in the detectable range. Particular examples of labels which may be used under the invention include fluorescein, rhodamine, dansyl, umbelliferone, Texas red, luminol, NADPH and α-β-galactosidase.

Production of PCV Proteins

The above described PCV proteins and active fragments, analogs and chimeric proteins derived from the same, can be produced by a variety of methods. Specifically, the proteins can be recombinantly produced as described herein. As explained above, these recombinant products can take the form of partial protein sequences, full-length sequences, precursor forms that include signal sequences, mature forms without signals, or even fusion proteins (e.g., with an appropriate leader for the recombinant host, or with another subunit antigen sequence for another pathogen).

Gene libraries can be constructed and the resulting clones used to transform an appropriate host cell. Colonies can be pooled and screened using polyclonal serum or monoclonal antibodies to the PCV protein.

Alternatively, once the amino acid sequences are determined, oligonucleotide probes which contain the codons for a portion of the determined amino acid sequences can be prepared and used to screen genomic or cDNA libraries for genes encoding the subject proteins. The basic strategies for preparing oligonucleotide probes and DNA libraries, as well as their screening by nucleic acid hybridization, are well known to those of ordinary

skill in the art. See, e.g., *DNA Cloning*: Vol. I, *supra*; *Nucleic Acid Hybridization*, *supra*; *Oligonucleotide Synthesis*, supra; Sambrook et al., *supra*. Once a clone from the screened library has been identified by positive hybridization, it can be confirmed by restriction enzyme analysis and DNA sequencing that the particular library insert contains a PCV protein gene or a homolog thereof. The genes can then be further isolated using standard techniques and, if desired, PCR approaches or restriction enzymes employed to delete portions of the full-length sequence.

Similarly, genes can be isolated directly from viruses using known techniques, such as phenol extraction and the sequence further manipulated to produce any desired alterations. See, e.g., Sambrook et al., *supra*, for a description of techniques used to obtain and isolate DNA.

Alternatively, DNA sequences encoding the proteins of interest can be prepared synthetically rather than cloned. The DNA sequences can be designed with the appropriate codons for the particular amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981) *Nature* 292:756; Nambair et al. (1984) *Science* 223:1299; Jay et al. (1984) *J. Biol. Chem.* 259:6311.

Once coding sequences for the desired proteins have been prepared or isolated, they can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning

and host cells which they can transform include the bacteriophage λ (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61 (*Streptomyces*), pUC6 (*Streptomyces*), YIp5 (*Saccharomyces*), YCp19 (*Saccharomyces*) and bovine papilloma virus (mammalian cells). See, Sambrook et al., *supra*; *DNA Cloning*, *supra*; B. Perbal, *supra*.

The gene can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the desired protein is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal peptide or leader sequence. If a signal sequence is included, it can either be the native, homologous sequence, or a heterologous sequence. Leader sequences can be removed by the host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

Other regulatory sequences may also be desirable which allow for regulation of expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

The control sequences and other regulatory sequences may be ligated to the coding sequence prior to

insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases it may be necessary to modify the coding sequence so that it may be attached to the control sequences with the appropriate orientation; i.e., to maintain the proper reading frame. It may also be desirable to produce mutants or analogs of the desired PCV protein. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are described in, e.g., Sambrook et al., *supra*; *DNA Cloning*, *supra*; *Nucleic Acid Hybridization*, *supra*.

The expression vector is then used to transform an appropriate host cell. A number of mammalian cell lines are known in the art and include immortalized cell lines available from the American Type Culture Collection (ATCC), such as, but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), Madin-Darby bovine kidney ("MDBK") cells, as well as others. Similarly, bacterial hosts such as *E. coli*, *Bacillus subtilis*, and *Streptococcus spp.*, will find use with the present expression constructs. Yeast hosts useful in the present invention include *inter alia*, *Saccharomyces cerevisiae*, *Candida albicans*, *Candida maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, *Pichia guillerimondii*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Insect cells for use with

baculovirus expression vectors include, *inter alia*, *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*.

Depending on the expression system and host selected, the proteins of the present invention are produced by culturing host cells transformed by an expression vector described above under conditions whereby the protein of interest is expressed. The protein is then isolated from the host cells and purified. If the expression system secretes the protein into the growth media, the protein can be purified directly from the media. If the protein is not secreted, it is isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

The proteins of the present invention may also be produced by chemical synthesis such as solid phase peptide synthesis, using known amino acid sequences or amino acid sequences derived from the DNA sequence of the genes of interest. Such methods are known to those skilled in the art. See, e.g., J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, 2nd Ed., Pierce Chemical Co., Rockford, IL (1984) and G. Barany and R. B. Merrifield, *The Peptides: Analysis, Synthesis, Biology*, editors E. Gross and J. Meienhofer, Vol. 2, Academic Press, New York, (1980), pp. 3-254, for solid phase peptide synthesis techniques; and M. Bodansky, *Principles of Peptide Synthesis*, Springer-Verlag, Berlin (1984) and E. Gross and J. Meienhofer, Eds., *The Peptides: Analysis, Synthesis, Biology*, *supra*, Vol. 1, for classical solution synthesis. Chemical synthesis of peptides may be preferable if a small fragment of the antigen in question is capable of raising an immunological response in the subject of interest.

Production of Antibodies

Proteins encoded by the novel viruses of the present invention, or their fragments, can be used to produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal, (e.g., mouse, rabbit, goat, horse, etc.) is immunized with an antigen of the present invention, or its fragment, or a mutated antigen. Serum from the immunized animal is collected and treated according to known procedures. See, e.g., Jurgens et al. (1985) *J. Chrom.* 348:363-370. If serum containing polyclonal antibodies is used, the polyclonal antibodies can be purified by immunoaffinity chromatography, using known procedures.

Monoclonal antibodies to the proteins and to the fragments thereof, can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by using hybridoma technology is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al., *Hybridoma Techniques* (1980); Hammerling *et al.*, *Monoclonal Antibodies and T-cell Hybridomas* (1981); Kennett et al., *Monoclonal Antibodies* (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500, 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the desired protein, or fragment thereof, can be screened for various properties; i.e., for isotype, epitope, affinity, etc. Monoclonal antibodies are useful in purification, using immunoaffinity techniques, of the individual antigens which they are directed against. Both polyclonal and monoclonal antibodies can also be used for passive immunization or can be combined with

subunit vaccine preparations to enhance the immune response. Polyclonal and monoclonal antibodies are also useful for diagnostic purposes.

Vaccine Formulations and Administration

The novel viral proteins of the present invention can be formulated into vaccine compositions, either alone or in combination with other antigens, for use in immunizing subjects as described below. Methods of preparing such formulations are described in, e.g., *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pennsylvania, 18 Edition, 1990. Typically, the vaccines of the present invention are prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in or suspension in liquid vehicles prior to injection may also be prepared. The preparation may also be emulsified or the active ingredient encapsulated in liposome vehicles. The active immunogenic ingredient is generally mixed with a compatible pharmaceutical vehicle, such as, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vehicle may contain minor amounts of auxiliary substances such as wetting or emulsifying agents and pH buffering agents.

Adjuvants which enhance the effectiveness of the vaccine may also be added to the formulation. Adjuvants may include for example, muramyl dipeptides, avridine, aluminum hydroxide, dimethyldioctadecyl ammonium bromide (DDA), oils, oil-in-water emulsions, saponins, cytokines, and other substances known in the art.

The proteins may be linked to a carrier in order to increase the immunogenicity thereof. Suitable carriers include large, slowly metabolized macromolecules

such as proteins, including serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art; polysaccharides, such as sepharose, agarose, cellulose, cellulose beads and the like; polymeric amino acids such as polyglutamic acid, polylysine, and the like; amino acid copolymers; and inactive virus particles.

The proteins may be used in their native form or their functional group content may be modified by, for example, succinylation of lysine residues or reaction with Cys-thiolactone. A sulfhydryl group may also be incorporated into the carrier (or antigen) by, for example, reaction of amino functions with 2-iminothiolane or the N-hydroxysuccinimide ester of 3-(4-dithiopyridyl propionate. Suitable carriers may also be modified to incorporate spacer arms (such as hexamethylene diamine or other bifunctional molecules of similar size) for attachment of peptides.

Other suitable carriers for the proteins of the present invention include VP6 polypeptides of rotaviruses, or functional fragments thereof, as disclosed in U.S. Patent No. 5,071,651, incorporated herein by reference. Also useful is a fusion product of a viral protein and the subject immunogens made by methods disclosed in U.S. Patent No. 4,722,840. Still other suitable carriers include cells, such as lymphocytes, since presentation in this form mimics the natural mode of presentation in the subject, which gives rise to the immunized state. Alternatively, the proteins of the present invention may be coupled to erythrocytes, preferably the subject's own erythrocytes. Methods of coupling peptides to proteins or cells are known to those of skill in the art.

Furthermore, the proteins may be formulated into vaccine compositions in either neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

Vaccine formulations will contain a "therapeutically effective amount" of the active ingredient, that is, an amount capable of eliciting an immune response in a subject to which the composition is administered. Such protection will be demonstrated by either a reduction or lack of symptoms normally displayed by an infected host and/or a quicker recovery time.

The exact amount is readily determined by one skilled in the art using standard tests. The protein concentration will typically range from about 1% to about 95% (w/w) of the composition, or even higher or lower if appropriate.

To immunize a subject, the vaccine is generally administered parenterally, usually by intramuscular injection. Other modes of administration, however, such as subcutaneous, intraperitoneal and intravenous injection, are also acceptable. The quantity to be administered depends on the animal to be treated, the capacity of the animal's immune system to synthesize antibodies, and the degree of protection desired. Effective dosages can be readily established by one of ordinary skill in the art through routine trials

establishing dose response curves. The subject is immunized by administration of the vaccine in at least one dose, and preferably two doses. Moreover, the animal may be administered as many doses as is required to maintain a state of immunity to infection.

Additional vaccine formulations which are suitable for other modes of administration include suppositories and, in some cases, aerosol, intranasal, oral formulations, and sustained release formulations. For suppositories, the vehicle composition will include traditional binders and carriers, such as, polyalkaline glycols, or triglycerides. Such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10% (w/w), preferably about 1% to about 2%. Oral vehicles include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium, stearate, sodium saccharin cellulose, magnesium carbonate, and the like. These oral vaccine compositions may be taken in the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders, and contain from about 10% to about 95% of the active ingredient, preferably about 25% to about 70%.

Intranasal formulations will usually include vehicles that neither cause irritation to the nasal mucosa nor significantly disturb ciliary function. Diluents such as water, aqueous saline or other known substances can be employed with the subject invention. The nasal formulations may also contain preservatives such as, but not limited to, chlorobutanol and benzalkonium chloride. A surfactant may be present to enhance absorption of the subject proteins by the nasal mucosa.

Controlled or sustained release formulations are made by incorporating the protein into carriers or vehicles such as liposomes, nonresorbable impermeable polymers such as ethylenevinyl acetate copolymers and Hytrel® copolymers, swellable polymers such as hydrogels, or resorbable polymers such as collagen and certain polyacids or polyesters such as those used to make resorbable sutures. The proteins can also be delivered using implanted mini-pumps, well known in the art.

The proteins of the instant invention can also be administered via a carrier virus which expresses the same. Carrier viruses which will find use with the instant invention include but are not limited to the vaccinia and other pox viruses, adenovirus, and herpes virus. By way of example, vaccinia virus recombinants expressing the novel proteins can be constructed as follows. The DNA encoding the particular protein is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the instant protein into the viral genome. The resulting TK recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

An alternative route of administration involves gene therapy or nucleic acid immunization. Thus, nucleotide sequences (and accompanying regulatory elements) encoding the subject proteins can be administered directly to a subject for *in vivo* translation thereof. Alternatively, gene transfer can be accomplished by transfecting the subject's cells or

tissues *ex vivo* and reintroducing the transformed material into the host. DNA can be directly introduced into the host organism, i.e., by injection (see International Publication No. WO/90/11092; and Wolff et al. (1990) *Science* 247:1465-1468). Liposome-mediated gene transfer can also be accomplished using known methods. See, e.g., Hazinski et al. (1991) *Am. J. Respir. Cell Mol. Biol.* 4:206-209; Brigham et al. (1989) *Am. J. Med. Sci.* 298:278-281; Canonico et al. (1991) *Clin. Res.* 39:219A; and Nabel et al. (1990) *Science* 249:1285-1288. Targeting agents, such as antibodies directed against surface antigens expressed on specific cell types, can be covalently conjugated to the liposomal surface so that the nucleic acid can be delivered to specific tissues and cells susceptible to infection.

Diagnostic Assays

As explained above, the proteins of the present invention may also be used as diagnostics to detect the presence of reactive antibodies of PCV in a biological sample in order to determine the presence of PCV infection. For example, the presence of antibodies reactive with the proteins can be detected using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation, etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith.

The aforementioned assays generally involve separation of unbound antibody in a liquid phase from a solid phase support to which antigen-antibody complexes are bound. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e.g., in membrane or microtiter well form); polyvinylchloride (e.g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

Typically, a solid support is first reacted with a solid phase component (e.g., one or more PCV proteins) under suitable binding conditions such that the component is sufficiently immobilized to the support. Sometimes, immobilization of the antigen to the support can be enhanced by first coupling the antigen to a protein with better binding properties. Suitable coupling proteins include, but are not limited to, macromolecules such as serum albumins including bovine serum albumin (BSA), keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art. Other molecules that can be used to bind the antigens to the support include polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and the like. Such molecules and methods of coupling these molecules to the antigens, are well known to those of ordinary skill in the art. See, e.g., Brinkley, M.A. *Bioconjugate Chem.* (1992) 3:2-13; Hashida et al., *J. Appl. Biochem.* (1984) 6:56-63; and Anjaneyulu and Staros, *International J. of Peptide and Protein Res.* (1987) 30:117-124.

After reacting the solid support with the solid phase component, any non-immobilized solid-phase

components are removed from the support by washing, and the support-bound component is then contacted with a biological sample suspected of containing ligand moieties (e.g., antibodies toward the immobilized antigens) under suitable binding conditions. After washing to remove any non-bound ligand, a secondary binder moiety is added under suitable binding conditions, wherein the secondary binder is capable of associating selectively with the bound ligand. The presence of the secondary binder can then be detected using techniques well known in the art.

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with a desired protein. A biological sample containing or suspected of containing anti-protein immunoglobulin molecules is then added to the coated wells. After a period of incubation sufficient to allow antibody binding to the immobilized antigen, the plate(s) can be washed to remove unbound moieties and a detectably labeled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample antibodies, the plate washed and the presence of the secondary binding molecule detected using methods well known in the art.

Thus, in one particular embodiment, the presence of bound anti-antigen ligands from a biological sample can be readily detected using a secondary binder comprising an antibody directed against the antibody ligands. A number of anti-porcine immunoglobulin (Ig) molecules are known in the art which can be readily conjugated to a detectable enzyme label, such as horseradish peroxidase, alkaline phosphatase or urease, using methods known to those of skill in the art. An appropriate enzyme substrate is then used to generate a detectable signal. In other related embodiments,

competitive-type ELISA techniques can be practiced using methods known to those skilled in the art.

Assays can also be conducted in solution, such that the proteins and antibodies specific for those proteins form complexes under precipitating conditions. In one particular embodiment, proteins can be attached to a solid phase particle (e.g., an agarose bead or the like) using coupling techniques known in the art, such as by direct chemical or indirect coupling. The antigen-coated particle is then contacted under suitable binding conditions with a biological sample suspected of containing antibodies for the proteins. Cross-linking between bound antibodies causes the formation of particle-antigen-antibody complex aggregates which can be precipitated and separated from the sample using washing and/or centrifugation. The reaction mixture can be analyzed to determine the presence or absence of antibody-antigen complexes using any of a number of standard methods, such as those immunodiagnostic methods described above.

In yet a further embodiment, an immunoaffinity matrix can be provided, wherein a polyclonal population of antibodies from a biological sample suspected of containing antibodies to the protein of interest is immobilized to a substrate. In this regard, an initial affinity purification of the sample can be carried out using immobilized antigens. The resultant sample preparation will thus only contain anti-PCV moieties, avoiding potential nonspecific binding properties in the affinity support. A number of methods of immobilizing immunoglobulins (either intact or in specific fragments) at high yield and good retention of antigen binding activity are known in the art. Not being limited by any particular method, immobilized protein A or protein G can be used to immobilize immunoglobulins.

Accordingly, once the immunoglobulin molecules have been immobilized to provide an immunoaffinity matrix, labeled proteins are contacted with the bound antibodies under suitable binding conditions. After any non-specifically bound antigen has been washed from the immunoaffinity support, the presence of bound antigen can be determined by assaying for label using methods known in the art.

Additionally, antibodies raised to the proteins, rather than the proteins themselves, can be used in the above-described assays in order to detect the presence of antibodies to the proteins in a given sample. These assays are performed essentially as described above and are well known to those of skill in the art.

The above-described assay reagents, including the proteins, or antibodies thereto, can be provided in kits, with suitable instructions and other necessary reagents, in order to conduct immunoassays as described above. The kit can also contain, depending on the particular immunoassay used, suitable labels and other packaged reagents and materials (i.e. wash buffers and the like). Standard immunoassays, such as those described above, can be conducted using these kits.

Accordingly, novel PCV isolates have been disclosed.